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NB100-297 Protocol

Protocol specific for TERT Antibody (NB100-297)

Western Blot Procedure

1) Resolve protein samples on a 7.5% SDS-PAGE.

2) Transfer proteins to PVDF membranes.

3) Block the membrane with 5% NFDM in PBST overnight at 4 degrees Celcius.

4) Dilute primary TERT antibody (NB 100-297) in PBST + 1% BSA.

5) Incubate membrane for 1 hour at RT.

6) Wash 3 times ten minutes on a shaker.

7) Incubate membrane with HRP conjugated secondary for 1 hour (RT), diluted in PBST + 1% BSA.

8) Wash 3 times ten minutes on a shaker.

9) Add ECL reagent, as per kit directions, and expose.

NOTE: This primary antibody is made in mouse and the isotype of the antibody is IgM.

Immobilization of Anti-hTERT anibody
All reagents were from the Seize Primary Mammalian IP Kit.

50 ml of mouse ascites (3.3 mg/ml) was diluted with 350 ml of coupling buffer and coupled to 400 ml of AminoLink Plus slurry per the manufactures instructions. Greater than 80% of the protein in the antibody solution were coupled to the beads.

Immunoprecipitation
hTERT was synthesized in rabbit reticulocytes using a pET vector and [35S]-methionine was used to allow visualization of the protein.

Beads were washed 2X with wash buffer (WB1): 20 mM Tris-acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM MgCl2, 100 mM potassium glutamate, 0.1% IGEPAL, and 1 mM DTT, then blocked twice with 250 mL of blocking buffer (20 mM Tris-acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM MgCl2, 100 mM potassium glutamate, 0.1% IGEPAL, 1 mM DTT, 0.5 mg/mL lysozyme, 0.5 mg/mL BSA, 0.05 mg/mL glycogen, and 0.1 mg/mL yeast RNA) for 15 min at 4 degrees C.

In between each washing and blocking step the beads were precipitated by centrifugation at 1500g and the supernatant was removed.

50 mL of blocking buffer was then mixed with the 50 mL RNA/protein sample and centrifuged at 17 000g for 10 min at 4 degrees C to remove any precipitates.

This supernatant was then added to the blocked beads and the samples were mixed on a rotary platform for 2 h at 4 degrees C.

Following mixing, the beads were washed three times with 325 mL of Wash Buffer #2 (20 mM Tris-acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM MgCl2, 300 mM potassium glutamate, 0.1% IGEPAL, and 1 mM DTT) and twice with 325 mL of TMG (10 mM Tris-acetate, pH 7.5, 1 mM MgCl2, and 10% glycerol).

The beads were precipitated by centrifugation at 1500g in between each wash and the supernatant was removed.

The beads were then resuspended in 1X SDS gel loading buffer containing 10 mM DTT and analyzed by SDS PAGE.

The immunoprecipitation was also performed on 1x10(7) A549 cells.

The beads were assayed by TRAP assay.

Results: IP of [35S]-labled hTERT resulted in 10% yield. This is the same efficiency we observed for anti-HA beads used to IP HA tagged hTERT. IP of telomerase from cells allowed isolation of beads that contained telomerase activity.

Conclusion: We successfully immobilized anti-hTERT antibodies on AminoLink beads using the Seize kit from Pierce. These can be used to immunopurify telomerase. The efficiency should be optimized, but the preliminary results are promising.

Protocol courtesy of Pamela K. Dominick and Michael B. Jarstfer from University of North Carolina, Chapel Hill.

Immunofluorescence

1. Cell growth and feeding for IF

   A. Seed cells in 4-chamber slides at 20,000 per chamber.

   B. Grow to medium confluence

   C. Feed with MCDB170+IP at -48 and -24 hr.

2. Fixing cells for IF

   A. Wash cells (~70-80% confluent) with 1XPBS.

   B. Fix slides each in 1:1 ice cold MEOH:acetone and place at -20C for 10 minutes.

   C. Store no more than 48 hr in 100% ethanol.

3. IF for hTERT

   A. Remove fixative/ethanol from slides.

   B. Add 1 ml 2N HCl to each chamber.

   C. Incubate for 20 minutes.

   D. Remove the HCl and neutralize with 1 ml 0.1 M Na-borate.

   E. Incubate for 5 minutes.

   F. Remove Na-borate and add 1 ml blocking buffer.

   G. Incubate for 2 hr at RT.

   H. Prepare NB 100-297 at indicated dilution.

   I. Incubate ON at 4C.

   J. Wash 4X5 min. in RT PBS.

   K. Add secondary (FITC conjugated rabbit anti-mouse IgM).

   L. Incubate at RT for 2 hrs.

   M. Wash 4X5 min. in 1X PBS.

N. Wash 5 min in 1X PBS with DAPI (1.5 ug/ml).

O. Rinse slides briefly on PBS.

P. Remove chambers from slides.

Q. Mount in Vectashield (Vector catalog # H1200) and observe.

Blocking buffer To 500 ml of 1X PBS:

A. 5 g fish gelatin (Sigma catalog #G7765)

B. 25 ml goat serum

C. 5 g BSA Filter through 0.2 u filter and store at 4C